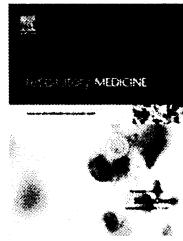


EXHIBIT A44



Talc pleurodesis: Evidence of systemic Inflammatory response to small size talc particles[☆]

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Summary

The mechanisms of the systemic response associated with talc-induced pleurodesis are poorly understood. The aim of this study was to assess the acute inflammatory response and migration of talc of small size particles injected in the pleural space.

Rabbits were injected intrapleurally with talc solution containing small or mixed particles and blood and pleural fluid samples were collected after 6, 24 or 48 h and assayed for leukocytes, neutrophils, lactate dehydrogenase, IL-8, VEGF, and TGF-beta. The lungs, spleen, liver and kidneys were assessed to study deposit of talc particles.

Both types of talc produced an acute serum inflammatory response, more pronounced in the small particles group. Pleural fluid IL-8 and VEGF levels were higher in the small particle talc group. Correlation between pleural VEGF and TGF-beta levels was observed for both groups. Although talc particles were demonstrated in the organs of both groups, they were more pronounced in the small talc group.

In conclusion, intrapleural injection of talc of small size particles produced a more pronounced acute systemic response and a greater deposition in organs than talc of mixed particles.

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Introduction

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Talc is the most extensively used agent for pleurodesis because of its wide availability and high rate of therapeutic success.¹ However, side effects associated with the intrapleural instillation of talc have been reported, particularly dyspnea,^{2–4} that may be severe and manifest as acute

respiratory distress syndrome in up to 9% of patients.^{1,2,5} The pathophysiology of the syndrome associated with talc pleurodesis is poorly understood and mainly involve diffuse pneumonitis, which is not observed with the use of other sclerosing agents.^{5–14} Several mechanisms have been proposed to explain talc-associated pneumonitis, including extension of the pleural inflammation to the lung parenchyma,¹⁵ the possible absorption of talc contaminants and the instillation of high doses of talc.^{1,14,16–18}

Another potential side effect of talc pleurodesis is the migration of talc particles from the pleural cavity to the systemic circulation,^{1,5,14,18–23} and it has been speculated that smaller talc particles (less than 10 µm) may more easily migrate to the bloodstream^{22–24} and contribute to the talc-associated pneumonitis.²⁵ Since the talc used for pleurodesis in Brazil presents a wide variation in particle size (6.4–50.5 µm),^{15,26} with 10% of the particles measuring less than 10 µm, we calculate that in pleurodesis induced with 5 g of talc about 500 mg of this agent consists of small particles, corresponding to a substantial amount instilled into the pleural cavity.

In the present study, we characterized the systemic and pleural inflammatory response of two types of talc, one with particles of variable size used traditionally for pleurodesis and one with particles of small size (less than 10 µm). Our hypothesis is that the smaller talc particles are responsible for the more intense acute systemic inflammatory response associated with talc pleurodesis.

Methods

The study was approved by the Ethics Committees for the analysis of research projects in humans and animals of Heart Institute (InCor), Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo, São Paulo, Brazil.

Talc particles

Asbestos-free talc [$Mg_6(Si_8O_{20})(OH)_4$] of "mixed size" (Magnesita, Brumado, BA, Brazil), routinely used for pleurodesis, which contains particles of varied size (mean size: 25.4 µm, range: 6.4–50.5 µm – only 10% of particles smaller than 6.66 µm), and small size talc, calibrated to 10 mm maximum diameter (Sigma-Aldrich Chemical Company, Milwaukee, WI, USA; mean size: 4.2 µm, range: 1.6–7.3 µm – with 50% of particles smaller than 6.41 µm) were used in this study.

The size of the talc particles was confirmed by granulometric analysis using laser diffraction (Malvern Instruments – Malvern, UK). The chemical composition of the talc particles was analyzed by X-Ray fluorescence spectrometry in the Laboratory of Environmental Pollution of the Medical College of the University of São Paulo. Both analyses showed no difference between both talc samples.

Intrapleural injections

Two groups of 15 New Zealand rabbits (2–2.5 kg) received 3-mL intrapleural injection of 400 mg/kg of either "mixed" or "small" talc diluted in sterile saline. One group of 10

rabbits received intrapleural saline as control. Blood samples were collected before and 6, 24 or 48 h after the instillation of the agents studied for the determination of biochemical, cytological and cytokine measurements.

Blood and pleural fluid samples were collected into tubes containing EDTA for cytological analysis and into dry tubes for the measurement of lactate dehydrogenase (LDH) and cytokines (IL-8, VEGF and TGF-β).

For the surgical procedure, the animals were anesthetized by intramuscular injection of 35 mg/kg ketamine (Cristália, São Paulo, SP, Brazil) plus 5 mg/kg xylazine (Bayer, São Paulo, SP, Brazil), followed by antisepsis with iodine solution (Rioquímica, São Paulo, SP, Brazil). Next, the different solutions were instilled with a 21-G needle (Becton–Dickinson, São Paulo, SP, Brazil) into the right pleural cavity as described in detail elsewhere.^{15,27–29} After instillation, the entire injection system was immediately removed to prevent the inadvertent entry of air into the pleural space. After the procedure, the animals were monitored for clinical evidence of pain (vocalization, dyspnea or agitation), and no analgesics were necessary.

The animals were sacrificed after 6, 24 or 48 h by a lethal injection of pentobarbital (60 mg/kg) through the marginal ear vein. To avoid inadvertent contamination of the materials with talc particles, the experiments were performed by the same examiner (EHG) who used talc-free gloves and each group was operated upon on different days.

Cytological and biochemical analysis

Blood and pleural fluid samples were collected into tubes containing EDTA for cytological analysis and into dry tubes for the measurement of lactate dehydrogenase (LDH) and cytokines. The cytology samples were stained with Leishman's stain for the determination of total leukocyte count and neutrophil percentage. LDH was measured by a kinetic UV method (normal serum levels ranging from 120 to 240 IU/L).

For the determination of cytokines, the supernatant of the samples was separated, aliquoted and stored in a freezer at –80 °C for subsequent analysis. IL-8 (OptEIA, rabbit IL-8 set; Pharmingen, San Diego, CA, USA), VEGF and TGF-β (R&D Systems, Minneapolis, MN, USA) were measured by enzyme-linked immunosorbent assay (ELISA) using the protocol suggested by the manufacturer and adapted for this study. Cytokines were quantified by the measurement of optical density in an ELISA reader (Power Wave, Bio-Tek, Winooski, VT, USA).

Tissue analysis

The thorax of the animals was removed en bloc and 50 mL 10% formaldehyde was injected into the trachea to prevent lung collapse and to facilitate histological analysis. The other organs (liver, spleen and kidneys) were removed and separately immersed in formalin for 48 h. For histological analysis by light microscopy, fragments were removed from the organs, embedded in paraffin, cut into 3-µm sections, and stained with hematoxylin-eosin (HE). Collection of the fragments was standardized in order to guarantee homogenous

samples. In the case of the lungs, the fragments were obtained from the inferior lobe. The other organs were sectioned transversely and fragments including the hilum of the organs were chosen as the most representative.

The presence of talc particles in the organs was analyzed by polarized light microscopy. The Leica Qwin image analysis program (Leica Q500IV Image Analysis System – Leica Imaging Systems Ltd., Heerbrugg, Switzerland), was used for microscopic analysis of the lungs ($\times 40$ magnification). Talc particles were quantified in 10 fields and the results are reported as the ratio between the areas occupied by the particles (detected by a colorimetric method) and the total area of the lung parenchyma, spleen, liver and kidneys.

Statistical analysis

The data were analyzed statistically using the SigmaStat software (San Raphael, CA, USA). Variables showing a normal distribution are reported as mean and standard deviation, and those showing no normal distribution are expressed as median and confidence interval. Blood and pleural fluid values were compared by analysis of variance (ANOVA) followed by the Tukey or Dunn multiple comparisons test when the difference was significant. Values obtained for small and mixed particle talc in pleural fluid were compared by the Student *t*-test. Pearson's (normally distributed data) or Spearman's (not normally distributed data) correlation test was used to determine the correlation between the pleural and serum parameters studied. The ratio of the area occupied by talc to the total lung or organs area was compared between groups by the Student *t*-test. A *p* value <0.05 was considered to be significant in all tests.^{30,31}

Results

Blood/serum

Leukocytes

Blood leukocytes were found to be significantly elevated early at 6 h after instillation of the two types of talc compared to the control group. After 24 h, leukocyte counts returned close to control values for the mixed talc, but remained significantly elevated in the small particle talc group. After 48 h, leukocyte counts returned to control values for both types of talc (Fig. 1).

Neutrophil percentage

As observed for leukocytes, the percentage of blood neutrophils was significantly elevated 6 h after instillation of the two types of talc compared to the control group. The neutrophil percentage returned close to control levels after 24 h and again increased after 48 h only in the small particle talc group (data not shown).

Lactate dehydrogenase

In the small talc group, LDH levels followed a similar pattern as that observed for neutrophil percentage, increasing over the first 6 h followed by a decrease after 24 h and then increasing again after 48 h. No difference compared to the control over time was observed for mixed particle talc (data not shown).

Interleukin-8

Serum IL-8 levels were significantly higher for the two types of talc when compared to control at all times. In addition, serum IL-8 tended to increase over time in the small particle talc group compared to the mixed talc group, with no statistical difference (Fig. 1).

Vascular endothelial growth factor

As observed for IL-8, serum VEGF levels increased during the first 6 h and remained elevated over time in the two groups when compared to the control group. No significant difference was observed for VEGF in the comparison of the two types of talc (Fig. 1).

Transforming growth factor- β

Higher serum TGF- β levels were observed for mixed talc at all time points, and for small particle talc group only after 48 h in comparison to control levels. When the two groups of talc were compared, the serum TGF- β levels were more pronounced in the mixed talc group at all time points (Fig. 1).

Pleural fluid (data not shown)

Leukocytes

Total leukocyte counts in pleural fluid were significantly higher in the mixed talc group compared to the small particle talc group at all time points, with these values tending to decrease over time. Leukocyte counts increased after 24 h in the small particle talc group and again decreased after 48 h.

Neutrophil percentage

Pleural fluid neutrophils showed an opposite behavior than that of leukocytes when comparing the two types of talc. The neutrophil percentage was significantly higher in the small particle talc group compared to the mixed talc group at all time points, showing an initial increase and a tendency to decrease over time.

Lactate dehydrogenase

LDH levels followed a trend similar to that observed for total leukocyte count in pleural fluid. Significantly higher levels were observed in the mixed talc group compared to the small particle talc group after 6 and 24 h, whereas after 48 h LDH levels were higher in the small particle talc group.

Interleukin-8

IL-8 levels in pleural fluid were significantly higher in the small particle talc group compared to the mixed talc group at all times.

Vascular endothelial growth factor

As observed for IL-8, VEGF levels in pleural fluid were higher in the small particle talc group than in the mixed talc group at all times.

Transforming growth factor- β

As previously stated for the serum levels, pleural TGF- β levels were higher in the mixed talc group than in the small particle talc group at all times.

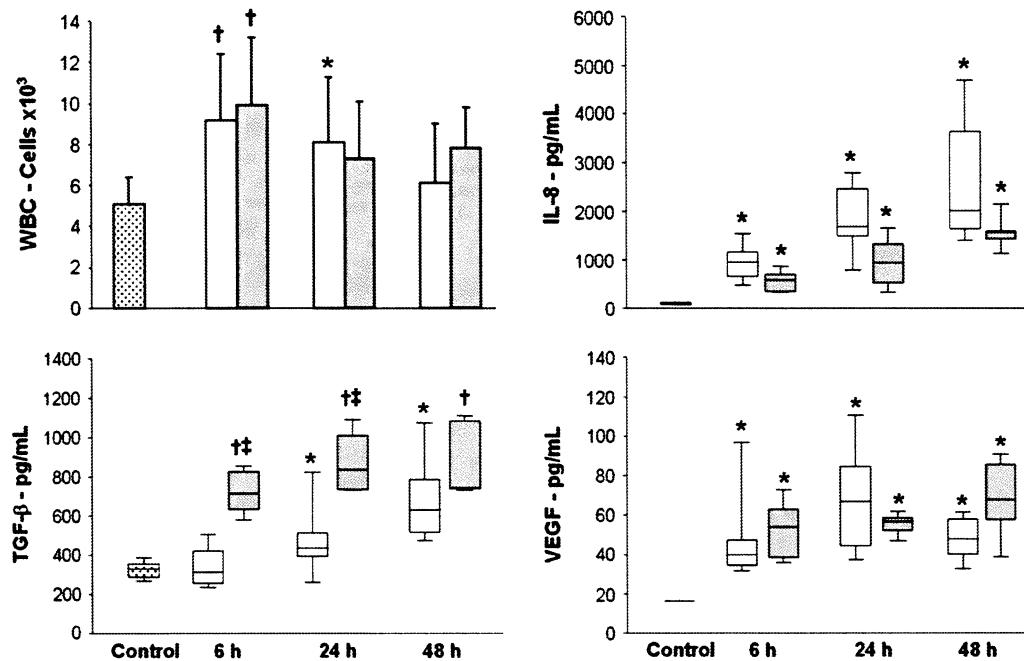


Figure 1 Serum inflammatory markers after intrapleural injection of talc. White blood cell count (WBC) and serum cytokines after intrapleural injection of small (ST- white bars) or mixed (MT- gray bars) particle talc. (1) WBC rise early at 6 h for both particles and remain elevated in the ST group until 24 h. After 48 h, WBC returns to normal for both groups. (2) IL-8 levels are higher than control at all times for both types of talc. (3) VEGF increases early at 6 h in both groups. (4) Higher TGF- β levels are seen in MT at all time points in comparison to ST ($^{\dagger}p < 0.001$ and $*p < 0.05$ vs. control; $^{\ddagger}p < 0.05$ MT vs. ST).

Correlations

Correlation between the pleural fluid and blood/serum parameters revealed a strong correlation only for TGF- β ($R = 0.80$; $p < 0.001$) in the small particle talc group, whereas in the mixed talc group a correlation was observed only for VEGF ($R = 0.74$; $p < 0.001$).

Comparison between the different parameters in each compartment (pleural fluid or blood) showed positive correlations only between VEGF and TGF- β in pleural fluid for both types of talc (small particle talc: $R = 0.94$; $p < 0.001$; mixed talc: $R = 0.72$; $p < 0.05$) (data not shown).

Tissue analysis

Lungs

In the small particle talc group, talc particles were observed adhered to the pleural surface and also in the lung parenchyma (alveolar spaces and septa) in the right lung (intrapleural injection side) (Fig. 2). In the left lung (contralateral to the injection side), talc particles were also detected in the alveolar septa but not in the lung parenchyma (Fig. 2).

In the mixed talc group, talc particles showed a heterogeneous distribution on the right pleural surface (side of injection), with particles rarely being observed in the lung parenchyma or alveolar septa (Fig. 2). In the left lung, talc particles were only visible in the alveolar septa (Fig. 2).

Quantification of talc particles in the parenchyma (ratio of the area occupied by talc to the total tissue area $\times 10^5$) revealed a significantly larger number of particles in both lungs in the small particle talc group compared to the mixed talc group (right lung: 71.1 ± 28.9 vs. 1.18 ± 0.69 ,

$p < 0.001$; left lung: 68.7 ± 42.4 vs. 1.13 ± 1.14 , $p < 0.001$). Of note, for both types of talc the particles deposition was similar in both lungs parenchyma (Fig. 3).

Spleen, liver and kidneys

Analysis of histological sections of the spleen parenchyma showed the presence of talc particles in the spleen pulp for both agents. Characteristically, the talc particles were found aggregated only in the red pulp, whereas the white pulp was preserved (Fig. 2).

A diffuse distribution of talc particles was also observed in sections of the liver parenchyma. As a particularity, the particles were characteristically located in perivasculär regions and therefore more demonstrated close to the hilä, centrolobular vessels and sinusoids (Fig. 2).

In the kidney, talc particles were predominantly detected in the medullary layer in both groups. Similar to the findings of other organs, talc particles were also detected in animals submitted to the intrapleural injection of mixed talc (Fig. 2).

Quantification of talc particles in the liver and kidneys showed a significantly larger number of particles in animals injected with small particle talc ($p < 0.05$). No significant difference was observed for the spleen (Fig. 3).

Correlation of the area ratio of talc in different organs

Analysis of the area ratio of talc in different organs revealed no positive correlations. In addition, no correlation between cytokine levels and the area ratio of talc was observed in any of the organs.

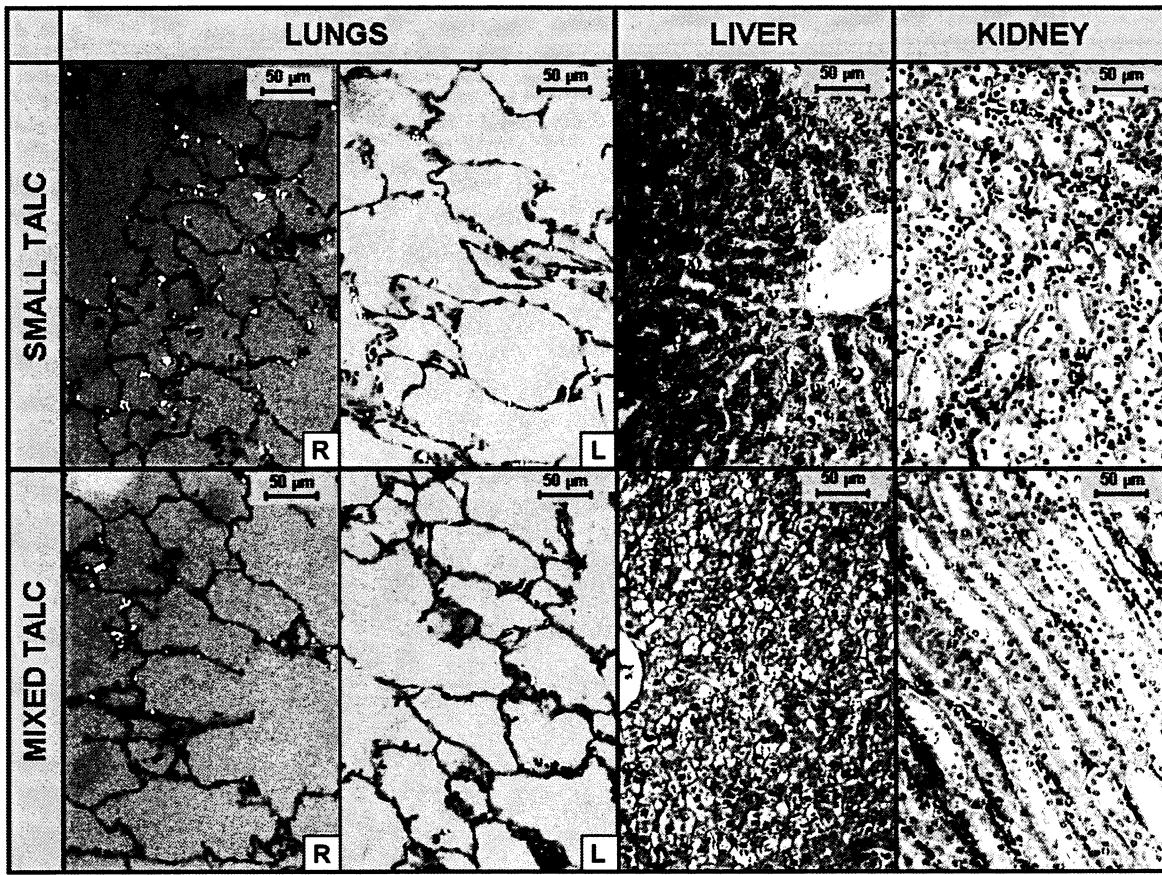


Figure 2 Photomicrographs of the lungs and organs – migration of talc particles. Photomicrographs of the lungs and organs. *Right lung*: talc particles are noted in the alveolar spaces and septa in both groups, with more particles in the ST (small talc) group than in the MT (mixed talc) group. *Left lung*: particles are seen only in alveolar septa with a greater number in the ST group. *Liver*: particles are characteristically located in the perivascular regions close to the hilum, centrolobular vessels and sinusoids. *Kidneys*: particles are noted predominantly in the medullar layer (HE and polarized light, $\times 200$).

Discussion

Talc, the agent most used for pleurodesis, is known to produce systemic effects that can culminate in respiratory insufficiency and even death. The mechanisms underlying these systemic effects are not completely understood. Experimental and clinical reports comparing the systemic effects of talc containing mixed and large particles (larger than $12 \mu\text{m}^{23}$ and $25 \mu\text{m}^{25}$) are available in the literature, but this is the first study that compares the systemic effects of talc containing mixed particles and talc containing particles smaller than $5 \mu\text{m}$.

Our findings indicate that both small and mixed talc injected intrapleurally in rabbits produce an acute systemic inflammatory response. However, small particle talc produced a more pronounced pleural and systemic response and resulted in greater particle deposition in the organs than mixed talc. Both types of talc caused an early increase in serum leukocyte, IL-8 and VEGF levels compared to control, but this increase was more marked in the small talc group. On the other hand, serum and pleural fluid TGF- β levels showed a more marked increase in the mixed talc group. Similar to the serum findings, IL-8 and VEGF levels in pleural fluid were also higher in the small particle talc

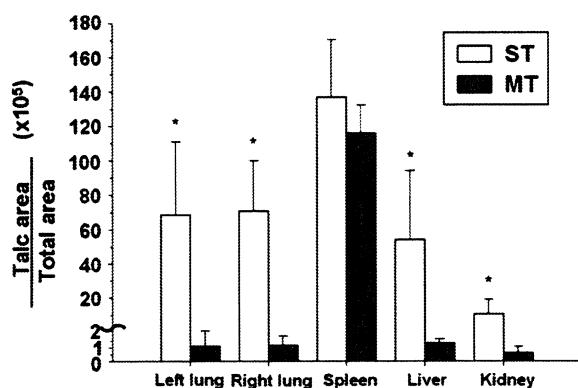


Figure 3 Particles deposition in the lungs and other organs. Ratio of talc area vs. total area ($\times 10^5$) in lungs, spleen, liver and kidney. Small talc (ST) has a higher ratio of deposition in the lungs, liver and kidney ($*p < 0.05$). No difference was observed in the spleen.

group. Notably, IL-8 levels increased early and decreased over time, and a correlation between pleural VEGF and TGF- β levels was observed for both types of particles. Histological analysis of tissues showed dispersion of both types of talc particles to all organs studied. However, analysis of the size of the particles in the organs demonstrated a greater tissue deposition in animals receiving small particle talc. Curiously, large amounts of talc particles were observed in both lungs, with a more pronounced deposition in the small talc group.

Literature evidence points to a possible migration of talc particles from the pleural cavity to the systemic circulation.^{1,5,14,18-23} In an experimental study, Ferrer et al.²³ demonstrated the presence of talc particles in different organs and suggested that small particles might be more easily absorbed by lymphatic stomata than larger particles. On the basis of the present observations, the question remains as to which mechanisms are involved in the systemic response to intrapleural talc instillation. One explanation for the systemic inflammatory response would be the direct passage of particles from the pleural space to the bloodstream, in agreement with the findings of previous studies.^{14,23} In this respect, the same mechanism might explain the systemic effects observed for mixed talc, since about 10% of the particles of this type of talc were smaller than 10 μm .

Talc particles migration mechanisms are still unknown. One possible mechanism is the lymphatic absorption of talc particles from the pleural cavity. However, by this mechanism it would be difficult to explain the presence of talc particles in the liver, spleen and kidneys, since these particles migrate from the pleural cavity to the systemic venous circulation through the lymphatic route and return to the lung where they probably are being removed. In this respect, it is also possible that the presence of talc in the alveoli may produce an intense local inflammation and changes in the alveolar-capillary barrier, causing reflow of particles to the systemic circulation. In this situation, we would expect diffuse lung injury which might even be the cause of the respiratory insufficiency observed in talc pleurodesis. However, histological analysis of the lungs of the animals submitted to intrapleural talc injection showed no intense acute pulmonary lesions that would support this mechanism.

A second mechanism would be that of an intense pleural inflammatory process leading to rupture of the pleural barrier caused by diffuse mesothelial injury. An important observation of the present study was that the particles found in the organs studied were smaller than 5 μm , a finding strongly suggesting that the systemic effects can be explained by the presence of small particles.

The leakage of inflammatory mediators from the pleural space to the bloodstream is suggested by our findings, as showed that in the pleural cavity IL-8 levels increased early and decreased over time, whereas the opposite was observed in blood. In contrast, VEGF levels tended to increase progressively over time in both pleural fluid and blood, a finding reflecting an increase in capillary permeability that contributes to both the formation of pleural fluid and the passage of smaller molecules from the pleural cavity to the blood circulation.

Comparison of the levels of inflammatory mediators in pleural fluid and blood showed a strong pleural-serum

correlation only for TGF- β levels in the small particle talc group and for VEGF in the mixed talc group, a finding suggesting a possible flow mechanism between the two compartments. However, these data alone do not allow us to infer that this is the only mechanism involved in the systemic response observed in talc pleurodesis. In addition to the increase in inflammatory mediators, we observed an acute increase in serum leukocytes and neutrophils in both talc groups coinciding with the acute pleural-serum inflammatory response, although no correlation was observed for these parameters.

A possible pro-inflammatory effect of TGF- β was demonstrated by the correlation that was observed between VEGF and TGF- β in pleural fluid. This fact was suggested in a previous study showing that TGF- β stimulates the production of VEGF by mesothelial cells and thus explains the increase in vascular permeability observed in pleurodesis inflammation.³² However, our findings showed that pleural VEGF levels were higher in the small particle talc group than in the mixed talc group, whereas pleural TGF- β levels were higher in animals injected with mixed talc, suggesting a possible overlap of both distinct inflammatory mechanisms, the leakage of inflammatory mediators from the pleural space and the systemic effects of talc particles deposition in organs. In agreement with these findings, previous experimental study from our group showed an increase of serum IL-8 and VEGF levels in rabbits after intrapleural instillation of mixed talc,²⁸ suggesting that these mediators may reflow from the pleural cavity to the systemic circulation and are involved in the genesis of the systemic effects observed in pleurodesis. In addition, Maskell et al. showed in a clinical trial that talc containing particles standardized at 15 μm produced a local and systemic inflammation when compared to tetracycline and talc particles larger than 25 μm .²⁵

Our data do not permit any conclusion as to whether the systemic cellular response is due to the flow of cells from the pleural cavity to the bloodstream or to a direct systemic cellular response to the presence of talc particles in the organs. However, we may speculate that the intense pleural inflammation caused by talc promotes the loss of integrity of the pleural barrier, permitting the free flow of cytokines and talc particles between the two compartments. In a second step, the presence of talc particles in tissues would trigger a local cellular response that contributes to the maintenance and amplification of the systemic inflammatory response. These considerations indicate that the mechanism underlying the systemic inflammatory response observed in talc pleurodesis still needs further investigation. However, it seems reasonable to assume that the size of the talc particles influences the inflammatory response and, possibly, the side effects observed in clinical practice. In this respect, future studies correlating the cellular response with the number of particles deposited in tissues may contribute to clarify this question.

Conclusions

In conclusion, talc containing particles of small size injected into the pleural space of rabbits produced a more

intense systemic and pleural inflammatory response than mixed particle talc. Particles of both types of talc were detected in the lungs, spleen, liver and kidneys of the animals studied. These data, together with those obtained in previous studies, suggests the need for the commercial development of a type of talc for universal use in pleurodesis that preferentially contains particles larger than 20 μm to permit the clinical use with greater safety and a lower risk of adverse systemic effects.

Conflict of interest

The authors have no conflicts of interests.

Ethics statement

The study was approved by the Ethics Committees for the analysis of research projects in humans and animals of Heart Institute (InCor), Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo, São Paulo, Brazil.

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